COMMUNICATION

Supplementary Information

Injectable Hyaluronic acid–based Hydrogel with Carbon Dots and Iron complex for Embolization

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Experimental

Materials

The following materials were used in this study: iohexol (TCI, Tokyo, Japan); coaxial microcatheter system (2.8 Fr, 130 cm, Progreat; Terumo Corporation, Tokyo, Japan); 1-(3-aminopropyl) imidazole (API), MTT, and ferric chloride ($FeCl_3$) (Sigma-Aldrich, St. Louis, MO, USA); dialysis membranes with a molecular weight cutoff (MWCO) of 100–500 Da (Spectrum Laboratories, Rancho Dominguez, CA, USA); minimum essential medium alpha (MEM-α), fetal bovine serum (FBS), and Dulbecco's phosphate-buffered saline (DPBS) (HyClone Laboratories, Logan, UT, USA); and the Image-iT™ LIVE Green Reactive Oxygen Species Detection Kit for fluorescence microscopy (Thermo Fisher Scientific, Waltham, MA, USA). HA (M_w = 260 kDa) was used as received.

Synthesis of CDs

APIO (API, iohexol CDs) were synthesized through a hydrothermal method using 1-(3-aminopropyl) imidazole (API) and iohexol as precursors. Briefly, iohexol and API were added to 15 mL of distilled water and stirred vigorously to ensure complete mixing. The precursor solution, with a molar ratio of approximately 6.5:1 (API:Iohexol), was then transferred to a Teflon-lined stainless steel autoclave and heated for 6 h at 180°C. After cooling to RT, the mixture was dialyzed against distilled water using a dialysis membrane with an MWCO of 100–500 Da for 3 days to remove unreacted components and by-products. The resulting solution was freeze-dried over 5 days to obtain dry APIO powder for further characterization and application.

Preparation of APIO/Fe complex

The APIO/Fe complex was prepared by coordinating Fe^{3+} with the surface functional groups of APIO CDs. Briefly, 500 mg of APIO powder and 270 mg of iron(III) chloride ($FeCl_3$) were dispersed in 5 mL of distilled water under constant stirring at RT for 2 h to facilitate complex formation. The resulting solution was subsequently dialyzed against distilled water using a dialysis membrane with an MWCO of 100–500 Da for 6 h to remove unbound ions and impurities. The purified APIO/Fe complex was then freeze-dried to obtain a dry powder for further use.

Characterization of APIO and APIO/Fe complex

The structural and physicochemical properties of APIO CDs were characterized using a range of analytical techniques. First, DLS analysis was performed using Zetasizer Nano ZS (Malvern Instruments, USA) at 25°C in an aqueous suspension to determine the hydrodynamic diameter and dispersion stability. Next, morphological evaluation was conducted by TEM (JEM-2100F, JEOL, Japan). For preparing the TEM specimens, APIO CDs were dispersed in distilled water, and a 20-μL aliquot was drop-cast onto a copper grid, followed by air-drying at RT. Crystallographic properties were analyzed using an X-ray diffractometer (Miniflex600, Rigaku, Japan) equipped with a Cu Kα source operating at 40 kV. Scans were acquired over a 2θ range of 3°–90° with a scan rate of 10° min^{-1}. Elemental composition of the APIO samples was determined by elemental analysis using a Flash 2000 CHNS analyzer (Thermo Fisher Scientific, USA). Fluorescence properties were evaluated using a spectrofluorophotometer (RF-6000, Shimadzu, Japan). Excitation–emission spectra were recorded with APIO and APIO/Fe complex dissolved in distilled water. The X-ray diffraction data of APIO and APIO/Fe complex were obtained using a Cu X-ray tube at 40 kV. The scan range was set from 2θ = 3°–90° with 10° min^{-1} as the scan speed. The iron contents in the APIO /Fe complex were quantified by inductively coupled plasma mass spectrometry (ICP-MS, iCAP Q, Thermo Scientific, USA). In addition, the iodine content of APIO were quantified using ICP-MS (Agilent 7900, Agilent Technologies, USA) at the Yonsei center for research Facilities.

Preparation of injectable hydrogel

To fabricate the injectable hydrogel, high-molecular-weight HA (M_w = 260 kDa) was first dissolved in DI water to a final concentration of 1.5 wt%. Separately, the APIO/Fe complex was prepared in distilled water such that the final iron ion concentration reached 200 mM. Before hydrogel formation, oligo-HA (oHA) was mixed with the APIO/Fe complex to improve the interaction between the nanocomposite and hydrogel matrix. Next, the 260-kDa HA solution was combined with the oHA–APIO/Fe mixture. The two components were thoroughly mixed using a three-way stopcock by repeatedly drawing and expelling the solution 50 times at RT, which resulted in the formation of a homogeneous injectable hydrogel. For biological evaluation, hydrogel extracts were prepared in accordance with ISO 10993 guidelines. Briefly, the injectable hydrogel was incubated at 37 °C for 24 h in the dialysis bag (100-500 Da), and the resulting extract was collected and used for cytotoxicity, intracellular ROS generation, and lipid peroxidation assays. For direct comparison, the extract concentration was adjusted to provide an iron concentration equivalent to that of the APIO/Fe complex.

CT and MRI contrast imaging

CT and MRI contrast properties of the APIO/Fe complex and its hydrogel formulation were evaluated under the following conditions. For CT analysis, samples containing 100 I mg/ml were prepared in PCR tubes and serially diluted using distilled water. CT imaging was performed using a Quantum GX µCT imaging system (PerkinElmer, Hopkinton, MA, USA), at Korea Basic Science Institute (Gwangju, Korea). The CT imaging system operated at 90 kV and 80 µA, with a field of view (FOV) of 72 mm, voxel size of 144 µm, and a scan time of 4 min. Hounsfield unit (HU) were obtained using the proprietary Quantum GX 3D viewer software.

For MRI analysis, APIO/Fe complex and APIO/Fe complex hydrogel, each containing 100 mM iron concentrations, were loaded into PCR tubes. The samples were then diluted using Distilled water. The measurement were conducted on a 9.4 T/21 cm horizontal-bore magnet (Agilent, USA) equipped with an Avance Neo Console (Bruker, Germany) and a 72 mm Bruker volume coil at the Korea Basic Science Institute (KBSI), Ochang. T_1 relaxivities were measured using a spin-echo (SE) sequence with variable repetition time (TR = 100-6000 ms) and TE = 5.21 ms, with a slice thickness of 2mm, FOV = 6 x 5cm^2, matrix = 128 x 128, and NEX = 1 (Total scan time : 52 min 26 s). T2 relaxivities were measured using a CPMG sequence, with TR = 10 s, 64 echoes (echo spacing = 10 ms), slice thickness = 2 mm, FOV = 6 x 5 cm^2, matrix = 256 x 214, and NEX = 1 (Total scan time : 35 min 40 s).

Methylene blue degradation assay

Methylene blue assay was used for monitoring the generation of hydroxyl radicals (•OH), indicative of Fenton activity. The pH of the APIO/Fe complex and APIO/Fe complex gel extract (100 mM iron concentration) was adjusted to 6 using NaOH or HCl under constant magnetic stirring. Then, 30 µL of 30% H_2O_2 was added to methylene blue and sample mixture. At the designated time intervals (0, 5, 10, 30, 60, and 120 min), 1000 µL of the sample was withdrawn and immediately quenched with 5 µL of 10% methanol to terminate the oxidative reaction. The samples were subsequently filtered after which the absorbance spectrum was measured using a UV–vis spectrometer.

EPR analysis

Hydroxyl radicals (•OH) were identified by EPR spectroscopy (EMXplus-9.5/12/P/L System). The EPR was adjusted to an iron concentration of 1 mM and treated with H_2O_2 and DMPO (5,5-dimethyl-1-pyrroline N-oxide). Each solution transferred into EPR tubes, and then the spectra were recorded.

Cell culture and incubation conditions

HepG2 (human hepatoblastoma cell line) cells were sourced from the Korean Cell Line Bank with the assigned number 88065 and cultured in MEM-α supplemented with 10% FBS and 1% penicillin–streptomycin. Cells were cultured at 37°C with 100% humidity and 5% CO_2 and subcultured in new media every 2 days.

Cytotoxicity assay

The cytotoxic effects of $FeCl_3$, APIO, and APIO/Fe complex were determined using the MTT assay on HepG2 cells. These cells were cultured in 48-well plates at a concentration of 2×10^4 cells/well, followed by incubation for 24 h.

Then, the cells were exposed to varying concentrations of $FeCl_3$, APIO, and APIO/Fe complex for 24 h. The plates were then washed twice with DPBS, and MTT reagents were added for an additional 4 h of treatment. Formazan crystals were solubilized with DMSO, and absorbance was measured at 570 nm using a microplate reader (Synergy H1, BioTeK, USA). The samples were dissolved in serum-free (SF) medium. All experiments were conducted in triplicate.

Intracellular ROS generation assay

In vitro ROS generation was measured using the dichloro-dihydro-fluorescein diacetate (DCFH-DA) kit. Briefly, HepG2 cells were seeded onto 25-mm sterile round cover glasses inserted in a 6-well cell culture plate at a density of 3×10^5 cells/well and incubated for 24 h at 37°C in 5% CO_2. Then, the medium was replaced with SF medium containing the APIO/Fe complex (1 mM Fe concentration) and incubated for 24 h. Next, the cells were incubated with DCFDA (0.2 mM) for 30 min and washed with DPBS, after which they were fixed in 4% paraformaldehyde solution at RT and stained with DAPI. The fluorescence intensity of DCFDA (λ_{ex} = 485 nm, λ_{em} = 535 nm) was detected using a confocal laser scanning microscope (CLSM). Fluorescence images were analyzed using the CLSM image browser software. Quantitative fluorescence intensity analysis was performed using the ZEN software (Carl Zeiss, Germany), and data were collected from three independent replicates (n = 3).

Rheological properties of the injectable hydrogel

A rotational rheometer (DHR-20, TA Instruments, USA) was used for determining the rheological properties of the injectable hydrogel incorporating the APIO/Fe complex. The frequency sweep test was conducted over a frequency range of 0.1–100 Hz to determine the viscoelastic behavior of the gel. To determine the gelation time of the APIO/Fe complex gel, the frequency was fixed as 1 Hz, and the storage and loss moduli were measured. The viscosity values of the HA mixture and APIO/Fe complex gel were measured at 1-Hz frequency. The relative complex viscosity value of the APIO/Fe complex gel was determined at 25°C and 37°C, with the strain and frequency fixed as 0.1% and 1 Hz, respectively. To explore the shear-thinning and self-healing capabilities, the recovery test was performed at a fixed frequency of 1 Hz. Alternating strain levels of 0.1% and 100% were applied cyclically to mimic mechanical stress and recovery. All rheological analyses of the injectable hydrogels and precursors were conducted with approximately 1 mL of the hydrogel sample.

Injectability analysis of the hydrogel

The photograph of the APIO/Fe complex gel injectability was confirmed by manually delivering the gel through a 1-mL syringe with needles of various gauge size (18, 20, and 22 G). Quantitative evaluation of injection force was performed using a universal testing machine (UTM, MTDI, Republic of Korea). The hydrogels were injected using a 1-mL syringe with needles of 18, 20, and 22 G. Measurements were obtained in triplicate at a flow rate of 1 mL min^{-1} using a syringe pump.

***In vitro* Stability Test of the APIO/Fe complex Hydrogel**

The long-term stability of the APIO/Fe hydrogel was evaluated under physiological-mimicking conditions. Independently prepared hydrogel samples (1 mL) were carefully overlaid with 1 mL PBS supplemented with 10% fetal bovine serum (FBS) under static conditions. At predetermined time points (1, 3, 5, 7, 9, 11, 13, and 15 days), the supernatant was gently removed from each sample, and the remaining hydrogel was subjected to rheological analysis. Complex viscosity was measured using a rotational rheometer equipped with a parallel-plate geometry. All measurements were performed at 1 Hz to assess the hydrogel's mechanical stability over time. The viscosity at each time point was normalized to the initial value (day 0) to determine the percentage of viscosity retention.

Coagulation assay

The blood coagulation effects of the APIO/Fe complex hydrogels were determined using fresh mouse whole blood that was collected in tubes containing sodium citrate as an anticoagulant and stored at 4°C. The prepared samples were placed into the tubes, whole blood was added, and then 0.2 M $CaCl_2$ was added to induce the formation of fibrin clots. After incubation for 5 min, 1 mL of DI water was gently added to each tube to lyse any erythrocytes that were not entrapped within the clot, and then the samples were incubated for 1 min. Next, the tubes were gently inverted and centrifuged. The resulting supernatant was transferred to a 96-well plate for measuring the absorbance at 540 nm. The BCI was calculated as follows: (absorbance_sample/absorbance_control) × 100 (%). Saline-treated samples served as the control group.

Embolization assay using a vascular model

The APIO/Fe complex hydrogel was injected into a vascular model using a 2.8-Fr catheter. The catheter was then carefully removed from the vascular model. All outlets except the inlet and outlet of BMF were sealed using gelatin. Subsequently, the BMFs were injected using a syringe pump with a flow rate of 1 mL min^{-1}. The BMFs were prepared by mixing together DI water, propylene glycol, D(+)-glucose, phenol red, and Alizarin Red S for 1 day.

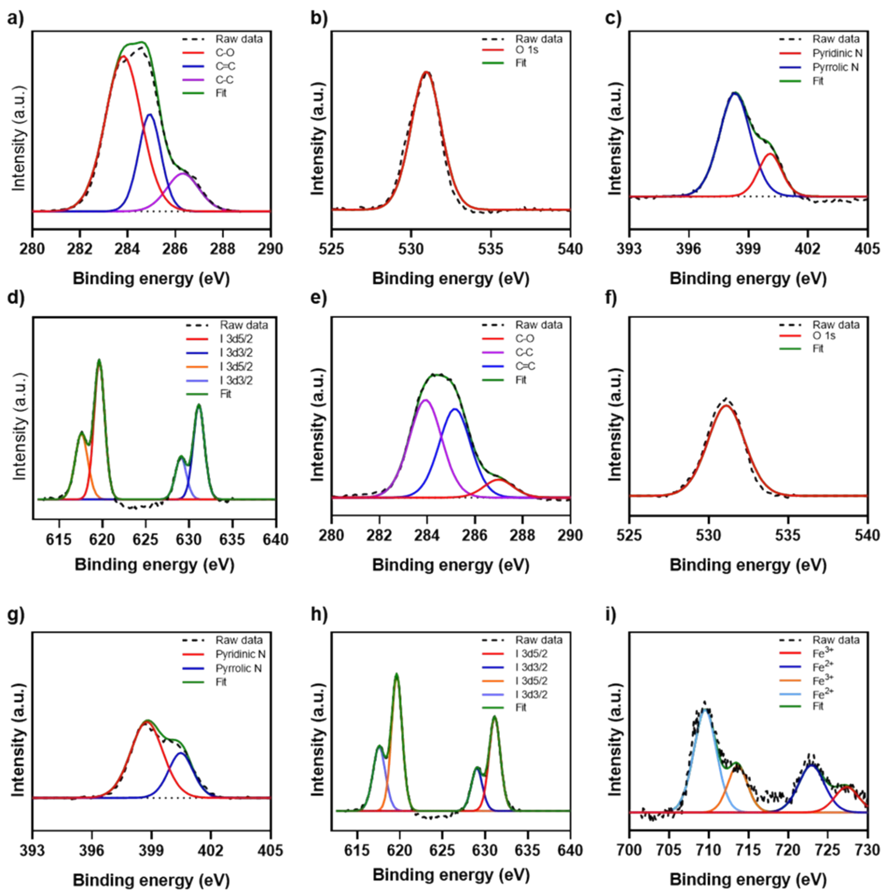

Figure S1. XPS analysis of APIO and APIO/Fe complex; deconvoluted high-resolution XPS spectrum of a) C 1s, b) O1s, c) N 1s, and d) I 3d of APIO. Deconvoluted high-resolution XPS spectrum of e) C 1s, f) O1s, g) N 1s, h) I 3d, and i) Fe 2p of APIO/Fe complex

	Fe^{3+} (A)	Fe^{2+} (B)	Fe^{3+} (C)	Fe^{2+} (D)
AREA	548.3	1095.2	832.7	2072.7
%	12.05	24.08	18.31	45.57
	Fe^{2+}		Fe^{3+}	
	Fe^{3+} (A)	Fe^{3+} (C)	Fe^{2+} (B)	Fe^{2+} (D)
%	30.36		70.91	

Figure S2. Calculated Fe^{2+} and Fe^{3+} ratios based on the integrated areas of the Fe 2p peaks (Figure S1 i).

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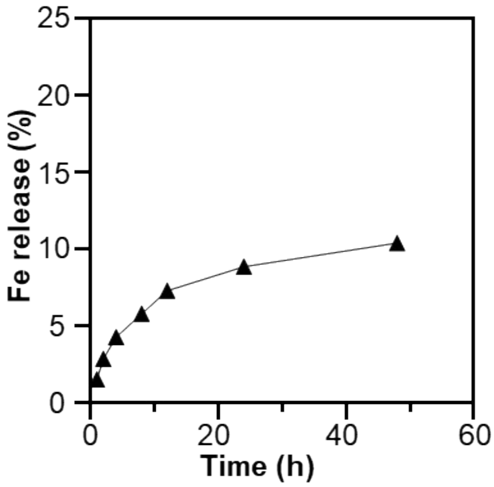

Figure S3. Iron ion release profile from the APIO/Fe complex hydrogel

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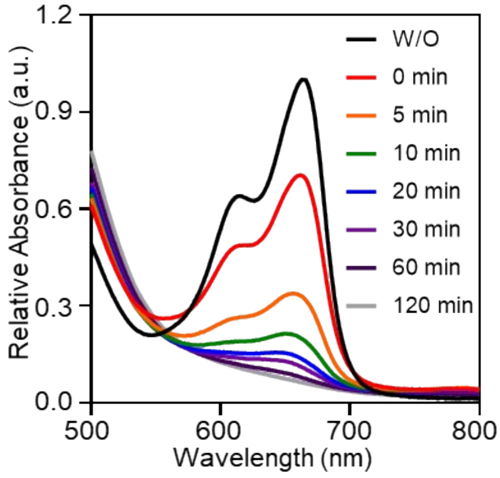

Figure S4. Fenton reaction assay via methylene blue degradation using APIO/Fe complex

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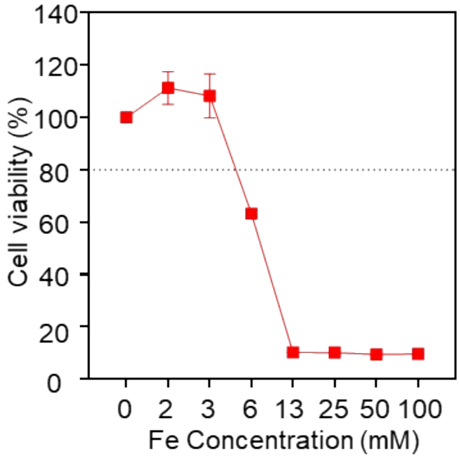

Figure S5. MTT assay of APIO/Fe complex

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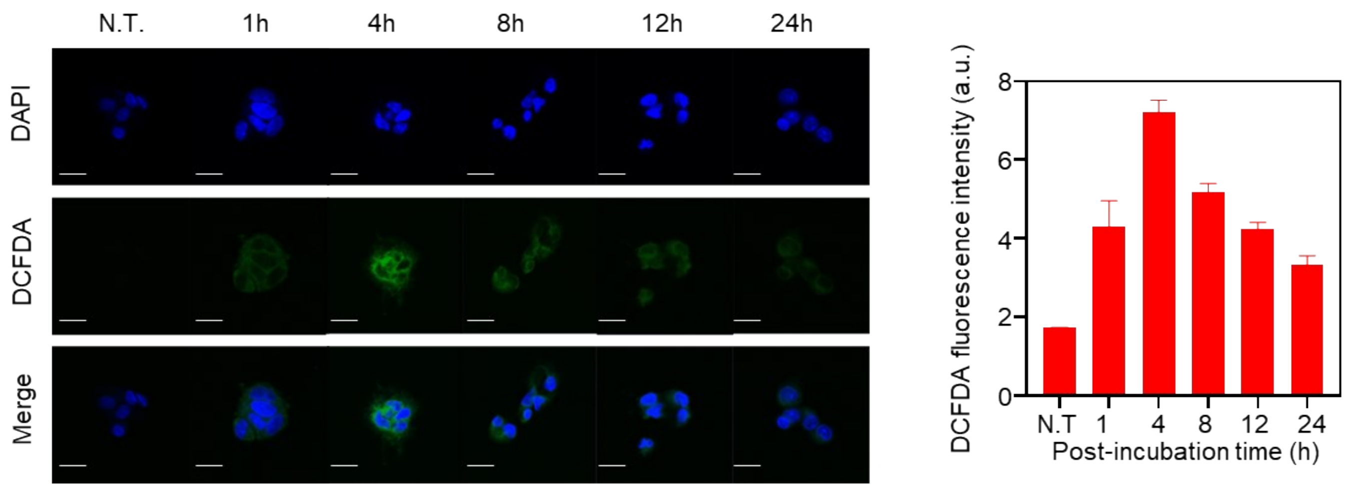

Figure S6. Detection of ROS by fluorescence intensity measurement using APIO/Fe complex.

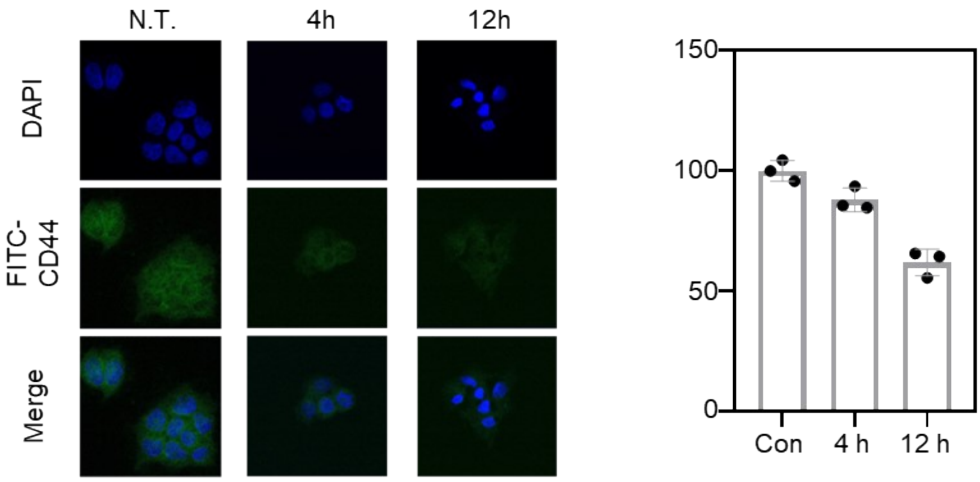

Figure S7. Detection of ROS by fluorescence intensity measurement using APIO/Fe complex

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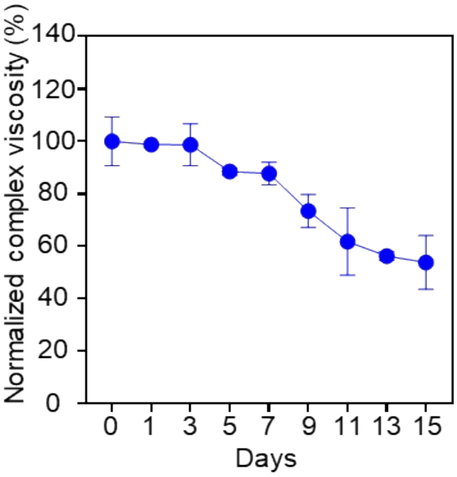

Figure S8. *In vitro* stability of the APIO/Fe hydrogel measured by complex viscosity over 15 days in PBS with 10% FBS at 37 °C. Values were normalized to day 0 (100%).

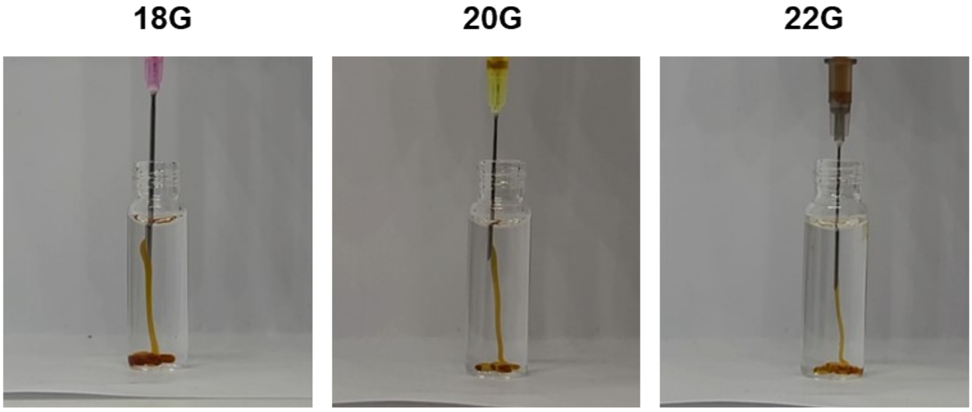

Figure S9. Photograph of the APIO/Fe complex gel injected using needles of different gauge sizes.

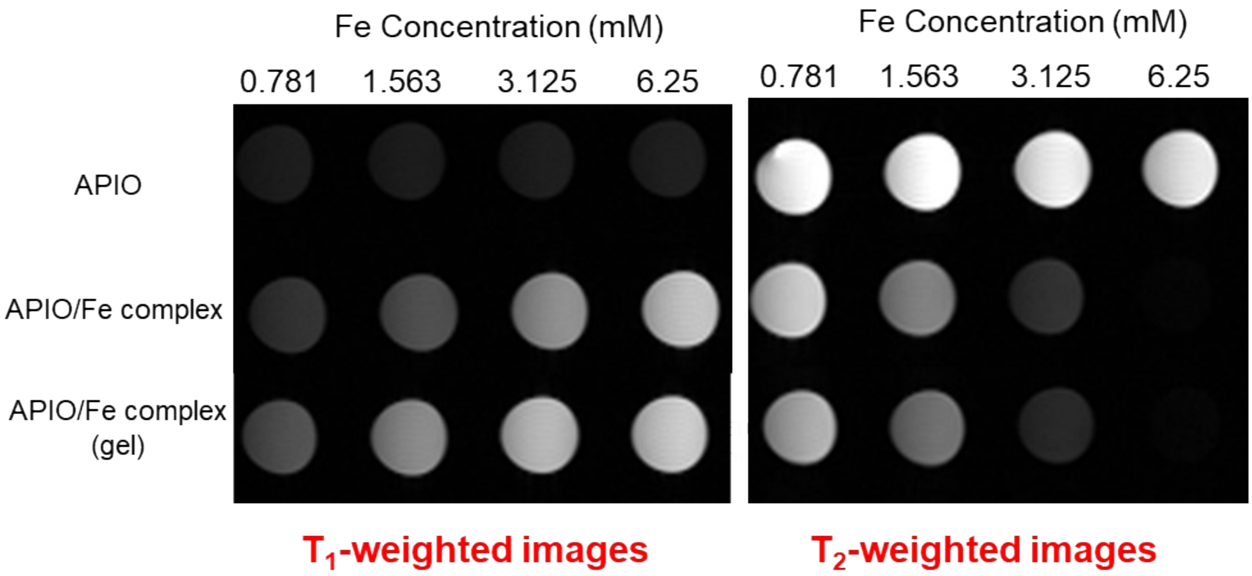

Figure S10. a) T1-weighted and b) T2-weighted MR images of samples.

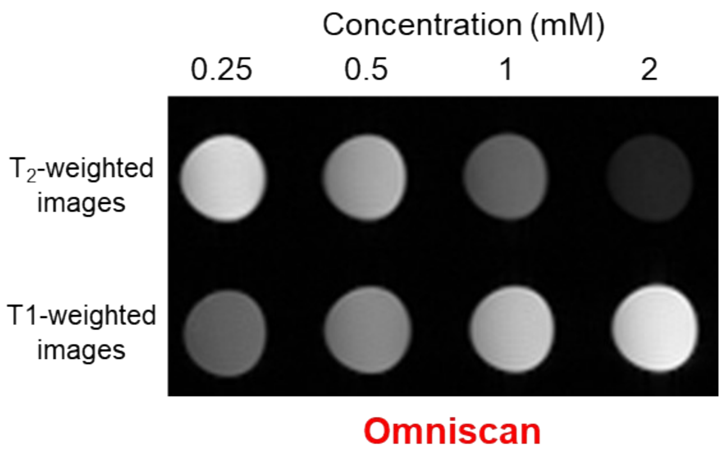

Figure S11. T_1- and T_2-weighted MR images of Omniscan

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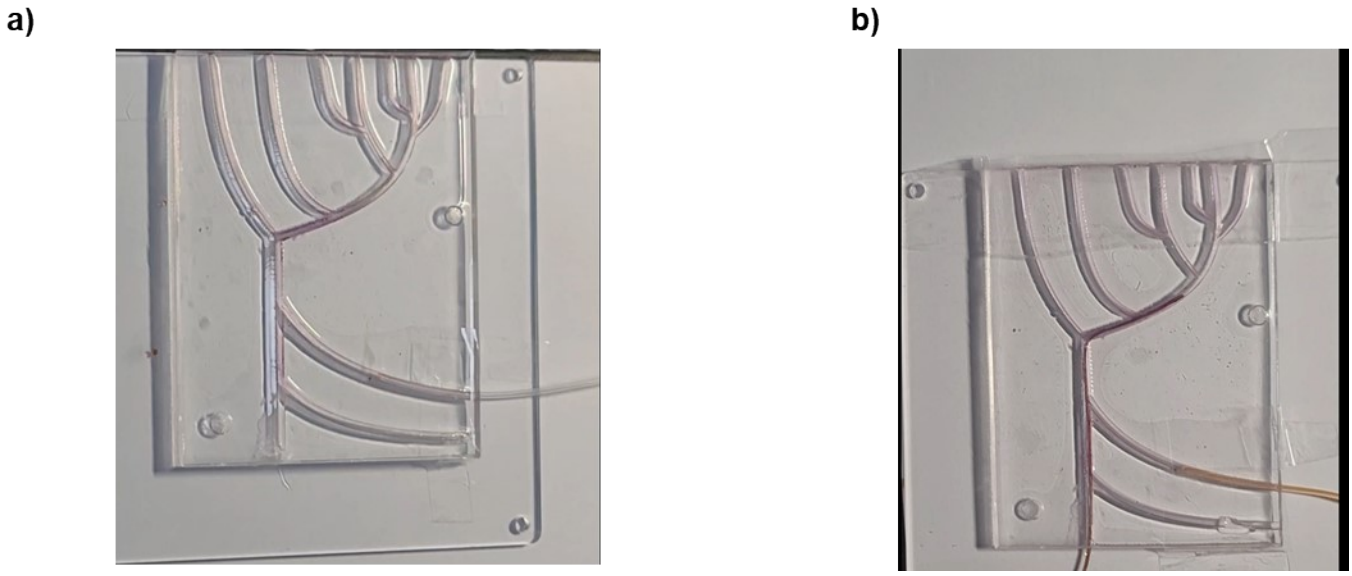

Figure S12. a) 3D vascular model without APIO/Fe complex gel (video) b) Embolization effect of APIO/Fe complex gel in 3D vascular model (video)